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Gilligan GR, Delgado AV, Burleson D, Cassidy R, Dubick MA, Bowman PD

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United States Army Institute of Surgical Research
3400 Rawley E. Chambers Ave.
Ft. Sam Houston, TX 78234

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14. ABSTRACT

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EVALUATION OF CYTOKINE SYNTHESIS IN HUMAN WHOLE BLOOD BY ENZYME LINKED IMMUNOASSAY (ELISA), REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR), AND FLOW CYTOMETRY

Gilligan G.R., Delgado A.V, Burleson D.,
Cassidy R., Dubick M.A., and Bowman P.D.

US Army Institute of Surgical Research,

Fort Sam Houston, TX 78234-6315

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Address correspondence to:
Dr. P.D. Bowman
US Army Institute of Surgical Research
3400 Rawley E. Chambers Ave., Bldg. 3611
Fort Sam Houston, TX 78234-6315
Ph (210) 916—4297
Fax (210) 916 2942

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ABSTRACT

Whole blood stimulated for 2 hours with small amounts of lipopolysaccharide (LPS, 10-1000 pg/ml) was used for evaluating methods for detecting cytokine expression in leukocytes. The anticoagulants heparin, citrate, and EDTA were examined for their influence on synthesis of tumor necrosis factor α (TNF-α), interleukin-lα, and -lβ~ (IL-lα and IL-1β), and interleukin-8 (IL-8). Cytokine secretion into blood was followed by enzyme-linked immunoassay (ELISA) and mRNA synthesis was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Leukocytes responsible for synthesis of cytokines were identified by immunolabeling of cell surface markers and intracellular cytokines followed by detection by flow cytometry. Translation of mRNA and secretion of cytokines into the plasma were affected by the method of anticoagulation. Use of heparin resulted in higher levels of cytokines than citrate or EDTA, Heparin interfered with RT-PCR detection of mRNA if it was not removed.

Within 30 min of LPS addition, significant increases in the synthesis of mRNAs for TNF-α, IL-lα and IL-l β, and IL-8 were detected by RT-PCR. Flow cytometry performed after intracellular labeling with anti-cytokine antibody indicated that monocytes were the most active leukocyte in cytokine synthesis while granulocytes also produced measurable amounts. Surprisingly, although the method of anticoagulation used had pronounced effects on the translation and secretion of cytokines, it had little influence on transcription of the respective gene.

INTRODUCTION

Understanding the interactions of multiple cytokine networks requires sensitive

techniques for determining cytokine concentrations and identifying the cells involved. Freshly drawn whole blood provides a simple and inexpensive source of leukocytes. Stimulation of cytokine synthesis by lipopolysaccharide has proven to be a valuable system for studying the production of cytokines by leukocytes. In previous studies, relatively high amounts of LPS, widely varying times of incubation, and dilution of whole blood with different amounts of medium have been used.[1-3] This last variable probably dilutes the lipopolysaccharide binding protein (LBP), which transports LPS to its cellular receptor, the CD14 molecule.[4] In addition, prior separation of leukocytes may lead to polymorphonuclear (PMN) activation.[5] Therefore, we have used undiluted, whole blood for this study.

Several lines of evidence indicate an important role for cytokines in the response to injury, in particular, after invasion by bacteria or exposure to bacterial products. While the cytokine ELISAs are the principal methods for estimating cytokine protein production, reverse transcriptase-polymerase chain reaction (RT-PCR) and cytokine flow cytometry[6] provide techniques that require relatively few cells for evaluating cytokine mRNA and protein synthesis of multiple cytokines in whole blood without prior purification.

The present study investigated the influence of different anti-coagulation procedures on the ability to detect cytokine synthesis and content by ELISA, RT-PCR, and cytokine flow cytometry following incubation with low doses of LPS.

MATERIALS AND METHODS

Whole Blood Treatment

Human blood was obtained following informed consent and was collected into Vacutainers (Becton-Dickinson, Rutherford, NJ) containing sodium citrate, potassium EDTA, or sodium heparin. One-milliliter aliquots of blood were placed in 1.5 ml microfuge tubes and

treated with various concentrations of lipopolysaccharide (LPS, *E. Coli,* Sigma, St. Louis, MO). Untreated blood served as control and all samples were incubated at 37°C on a platform rocker. When isolated mononuclear leukocytes were required, 2 volumes of blood were layered over 1 volume Polymorphprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 500 g for 30 minutes in a swinging bucket rotor. The mononuclear band at the interface was removed, washed with phosphate buffered saline (PBS), counted and lysed with TRI reagent (Molecular Research Center, Cincinnati, OH).

ELISA

After incubation for the appropriate time, one milliliter of blood was centrifuged in an Eppendorf fixed-angle rotor microfuge at 4°C and 8,000 g. Plasma was removed and stored at -70°C prior to measurement of cytokines by ELISA. To assay for intracellular cytokines (IL-lα and IL-1ß), blood was layered over Polymorphprep (Nycomed Pharma AS, Oslo, Norway) in a blood-to-Polymorphprep ratio of 2:1 to separate leukocytes. The blood was centrifuged at 500 g for 30 minutes in a swinging bucket rotor. The mononuclear leukocyte band at the interface was removed with a pipette, pelleted by centrifugation at 5,000 g and washed with sterile PBS. The cells were then reconstituted in 100 µl of deionized, sterile water and snap frozen to -80°C. They were then frozen and thawed three times to release intracellular constituents; particulates were removed by centrifugation and the resulting supernatant was stored at -80°C prior to measurement by ELISA.

ELISA kits were obtained from R & D Systems (R&D Systems, Minneapolis, MN) or Intergen (Purchase, NY) and used according to the manufacturers' instructions. The optical density for each well was determined with a microplate reader (Molecular Devices, Sunnyvale, CA). The amount of cytokine was determined by reference to a standard curve utilizing the

Softmax™ (Molecular Devices) data reduction software. The best-fit curve was a four-parameter logistics equation with a correlation coefficient of at least 0.98.

RNA Isolation and RT-PCR

Immediately following the end of incubation with LPS, 250 µl of blood were treated with TRI Reagent® LS (TRI-LS) (Molecular Research Center, Inc. (MRC), Cincinnati, OH) and total RNA isolated per manufacturer's instructions. If the same blood sample for ELISA was used for RT-PCR, an equal volume of saline was added to the packed cells before addition of TRI Reagent LS. RNA was dissolved in 10 µl of Formazol (MRC) and quantified fluorometrically in a Millipore Cytofluor according to Schmidt and Ernst.[7] 0.1 µl of RNA unknown or standard was added to 100 µl of solution TE buffer containing a 1/10,000 dilution of SYBR Green II (Molecular Probes, Inc., Eugene, OR). About 5 µg of total RNA was usually obtained from each 250 µl sample. The quality of the mRNA was determined by running 1.0 µg on a 1% agarose gel. Gels were stained in SYBR Green II diluted 10,000-fold in deionized water. Densitometry analysis was performed on the 18S and 28S bands, and compared to 1 µg of RNA ladder of known mass (Gibco BRL, Grand Island, NY). Only RNA exhibiting an undegraded pattern was used for RT-PCR.

cDNA was produced with You-Prime-First-Strand Synthesis Beads (Pharmacia Biotech, Inc., Piscataway, NJ). When heparinized whole blood was used, mRNA was reprecipitated with LiCl following isolation of total RNA. LiCl was added to a final concentration of 2.5 M after resolubilization of the RNA in 50 µl of water. One microgram of total RNA was primed with random hexamers (Gibco BRL) in the first strand reaction. PCR was performed in an MJ Research Thermal Cycler and Hot Bonnet (MJ Research, Waterstown, MA) without oil overlay. A typical PCR reaction contained concentrations of 200 µM of each deoxynucleotide

triphosphate, 1.5 mM $MgCl_2$, 400 nM primers, and 0.3 units of Taq polymerase, in a total volume of 10 µl. Taq polymerase, PCR buffer, and $MgCl_2$ were purchased from Gibco BRL, and deoxynucleotide triphosphates, from Sigma. Sequences for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), IL-8,and TNF-α were amplified with primer pairs and a primer specific mimic for each marker (Clontech Corp., Palo Alto, CA). No mimic was available for IL-1β. The mimic serves as an internal control for PCR and a target to mimic ratio is presented for these data. IL-1β was amplified semi-quantitatively followed by gel densitometry analysis. By running several reactions for each sample, each one a few cycles longer, and using several dilutions of template, it was possible to insure that data were acquired during the exponential phase of PCR.

Cytokine Flow Cytometry

Flow cytometry was utilized to determine the cell types responsible for the synthesis of mRNA and product detected by ELISA. Whole blood for flow cytometry was incubated with brefeldin A (Sigma, 10 µg/ml) immediately after addition of LPS (100 or 1000 pg/ml). The positive control consisted of treating one group with phorbol-12-myristate 13-acetate (PMA) (Sigma Chemical Co, 1 µg /ml) and ionomycin (Sigma Chemical Co, I µg /ml). Isotypic controls for both the intracellular cytokine antibodies and the cell surface markers were performed for each of the corresponding fluorochromes. Each group was incubated at 37°C, for 2 hr on a platform rocker. 100 µl of blood from each group were added to a 12 X 75 mm polystyrene tube containing cell surface markers: either anti-CD3 fluoroscein (FITC) as a T-cell marker, or anti--CD19 tricolor (TC) as a B-cell marker or anti-CD14 FITC as a monocyte marker, and anti-CD-16 TC as a granulocyte marker. All monoclonal antibodies were purchased from Caltag Laboratories (Burlingame, CA). After mixing, the tubes were incubated at 22°C in the dark for

15 min. Two milliliter of Fluorescence Activated Cell Sorter (FACS) lysing solution (Becton Dickinson, Sunnyvale CA) was added to each tube, vortexed, and incubated for 10 min at room temperature. Tubes were centrifuged at 200 g for 5 min. To the remaining pellet, 100 µl of Fix & Perm, Reagent A (Caltag, S. Burlingame, CA) was added and incubated at 22°C for 15 min. Two milliters of Hank's balanced salt solution (HBSS) were added to each tube before centrifugation at 200 g for 5 min. To permeabilize cell membranes, 100 µl of Fix and Perm, Reagent B (Caltag) was added to the pellet, followed by 10 µl of the appropriate intracellular cytokine monoclonal antibody. The following monoclonal antibodies were used: anti-IL-1β PE, anti-TNF-α PE (Caltag) or anti-IL-8 (Becton Dickinson). The cells were vortexed and incubated at 22°C in the dark for 15 min. Samples were washed with 2 ml of HBSS and centrifuged at 200 g for 5 min. The supernatant was aspirated, and the pellet was resuspended in 400 µl of 2% paraformaldehyde. Specimens were stored in the dark at 4°C for 24 hr until flow cytometry analysis. Flow cytometry acquisition was performed using a Becton Dickinson FACSCalibur flow cytometer and 10,000 events were acquired.

Analysis of the samples was done using the Becton Dickinson software CELLQuest 3.0.1. Data were analyzed two different ways. First, the population of interest, FL-1 or FL-3, was gated against side scatter and the percent positive cells over the isotypic control was calculated and expressed as percent positive. Likewise, the population of interest was gated using FL-1 or FL-3 against side scatter. The mean FL-2 fluorescence channel for IL-1β, TNF-α and IL-8 was calculated from a 256 linear scale, and all the control background values were subtracted from both the LPS and PMA treated samples.

Measurement of extracellular calcium

To determine the effects of various anticoagulants on free calcium levels in plasma,

serum derived from whole blood anticoagulated with citrate, EDTA, or heparin was analyzed for free calcium by the cresolphthalein complexone method[8] with the Boehringer-Mannheim/ Hitachi ISE system.

Statistical Treatment

Blood samples from three different male donors were utilized for these experiments. The data presented for ELISA and RT-PCR are representative experiments, while the data for cytokine flow cytometry is the average of three experiments. All data were expressed as the mean +/- standard error of the mean (SEM). Measurements from triplicate samples per treatment were averaged and analyzed using one-way analysis of variance (ANOVA) for comparison of treatments. Differences between treatments were considered significant if $p \leq 0.05$. Coefficients of variation were used to assess reproducibility where indicated.

RESULTS

ELISA Determination of Release of TNF-α, IL-1β and IL-8 as a function of anticoagulant

After 2 hrs of incubation with LPS at 37°C, large amounts of TNF-α and IL-8 were released into the plasma, in contrast to the very small amounts of IL-lα (data not shown) and IL-lβ which were barely above baseline (Figure 1). The amount of cytokine released was influenced by the anticoagulant. In all cases, the greatest cytokine release was detected when heparin was used, while the least amount was measured after anticoagulation with EDTA (Figure 1).

A comparison of anticoagulation method with extracellular calcium concentration is shown in Table I. Disturbances in extracellular calcium by EDTA and citrate may account for their influence on cytokine synthesis and release.

RT-PCR

In order to obtain quantitative data with RT-PCR, the starting mRNA was normalized to a common, invariant mRNA, so that equal amounts of mRNA were being compared. This was accomplished by normalizing all samples to the mRNA for the moderately expressed housekeeping function glyceraldehyde-3-phosphate dehydrogenase. To control for variation in the PCR process, a mimic was included. The mimic is a piece of DNA of known quantity that is amplified by the same primer pairs as the target, but which runs with different mobility in the gel.

Initial attempts to use RT-PCR with RNA derived from heparinized whole blood were unsuccessful due to copurification of heparin with the RNA. Interference by heparin with both reverse transcription and with PCR is indicated in Figure 2. Heparinized whole blood showed no PCR product for glyceraldehyde-3 -phosphate dehydrogenase target site (arrows) and a greatly reduced amplification of the mimic (pointers). When mononuclear cells were isolated prior to the

RNA extraction so that heparin was removed with the plasma, RT-PCR detection of gene expression by was not inhibited (Figure 2). Heparin inhibition of RT-PCR could be eliminated by re-precipitating isolated RNA with 2.5 N lithium chloride (LiCl).[9] For subsequent RT-PCR studies, this method was used.

The dose dependence of LPS stimulation of TNF-α, IL-lα, and IL-lβ after a 1 hr incubation at 37°C is illustrated in Figure 3. mRNA production was titrated against 10-fold dilutions of LPS at 0, 10 pg/ml, and 100 pg/ml in whole blood as illustrated with IL-8 (Figure 3c). Message was not significantly elevated above baseline as shown by the target/mimic ratio at LPS concentrations of 1.0 pg/ml blood, but at 10 pg/ml, message was significantly higher than control for IL-lα, and IL-lβ. This differential transcription of message for these cytokines (under the influence of 10 pg/ml LPS) occurred although there was no elevation of product in the plasma at 1 hr at this concentration. Ten pg/ml was the threshold level for response for mRNA up regulation for all cytokines measured, and induction at 100 pg/ml was significantly greater than that at 10 pg/ml compared to control (0 pg LPS).

IL-lα message showed significant elevation after a 1 hour stimulation with 100 pg/ml LPS (Fig 3). 10^{-3} attomoles of mimic (corresponding to 600 molecules) were used in each 10 µl reaction. Starting mimic copy number was 10-fold lower than that used in the dose-response IL-8 PCR, and thus the target in the cDNA was estimated to be roughly ten times less concentrated. The reaction was run for 33 cycles. Three extra cycles were used to increase band intensity in the gel about five fold while maintaining the reaction within the exponential phase of the amplification.

Using 10^{-2} attomoles of mimic per reaction, 10 pg/ml LPS-treated blood demonstrated a significant increase in TNF-α mRNA transcription and at 100 pg/ml of LPS, the yield was

significantly higher. The PCR spanned 30 cycles (Fig 3).

Although no mimic was available for IL-lβ, a qualitative difference was still observed when reactions were performed in triplicate. There was no product at control, while 10 pg/ml and 100 pg/ml showed increasing levels of message with dose. This reaction was amplified with 30 PCR cycles (Fig 3).

The TNF-α, and IL-8, and PCR products correlated well with the cytokine release into the plasma in non-LPS treated and LPS-stimulated samples as measured by ELISA. This relationship did not hold for IL-lα and IL-lβ, however; very little product was obtained in the medium although a strong PCR signal was generated at 1 hr. Apparently, processing of the IL-lα and IL-lβ, required additional steps. For short incubations with LPS (1 hr), the anticoagulant used did not have a significant influence on the amount of mRNA detected by RT-PCR for the 4 cytokines (Figure 4). Disturbance in extracellular calcium by the method of anticoagulation affected synthesis and release more than transcription.

Time dependent response of cytokine mRNA

To estimate the time required for cells to respond to LPS, whole blood was incubated with LPS at 30 min and 1 hour. In comparison to other cytokines studied, TNF-α mRNA amplified most strongly in competitive PCR in comparison to a known concentration of mimic at 30 minutes. Thirty PCR cycles were performed with 10^{-2} attomoles of mimic (corresponding to about 6000 molecules) in each 10 μl reaction. IL-8, also performed with 10^{-2} attomoles of mimic in each 10 μl reaction, showed mRNA transcription elevation at this time point in relation to controls, but it was not elevated to the same degree as TNF-α. These results are shown in Figure 5.

Cellular Identification and Intracellular Cytokine Determination by Flow Cytometry

Three-color flow cytometry was performed on cells from whole blood to obtain information about the cells responsible for the synthesis and secretion of TNF-α, IL-Lβ, and IL-8. Based on specific antibody binding to cell surface markers and the amount of side scatter, leukocytes could be defined as monocytes, granulocytes, T-cells or B-cells (Figure 6).

Intracellular staining for cytokines indicated that the principal cell types responsible for the synthesis of TNF-α, IL-Lβ, and IL-8 were monocytes and granulocytes (Figure 7). Intracellular accumulation of cytokines and identification by flow cytometry also indicated that lower numbers of cells were positive for all three cytokines when whole blood was anticoagulated with either citrate or EDTA (data not shown). The frequency of cytokine producing cells anticoagulated with heparin and stained for T-cells, B-cells, monocytes and granulocytes and TNF-α, IL-β and IL-8 is shown in Figure 8. Forty percent of monocytes showed a strong signal for all three cytokines, and 11 percent of granulocytes were positive. Since about 70 percent of leukocytes were granulocytes they should be the largest contributor to the cytokines measured. The increase in mean channel fluorescence was about 2.5 times greater in the monocytes than the granulocytes, indicating that the monocytes produced more cytokine on a per cell basis.

DISCUSSION

Whole blood culture provides a relatively simple and clinically relevant model for analysis of synthesis and release of proinflammatory cytokines. Here, we have defined conditions for use of ELISA, RT-PCR and cytokine flow cytometry for estimating cytokine production after stimulation by physiologically relevant concentrations of LPS. Cytokines assayed were TNF-α, IL-lα, IL-1β, IL-6 and IL-8. Messenger RNA was elevated for all of these

cytokines and significant amounts of TNF-α and IL-8 were secreted. IL-lα and IL-lβ mRNA were translated, but little product was secreted. Since no production of IL-6 mRNA or product stimulated by LPS were detectable at the short time points used, it was not investigated further.

The importance of the method of anticoagulation on the synthesis and translation of mRNA for four cytokines was investigated. In measuring cytokine levels using ELISA, cytokine release was noticeably higher from heparin anticoagulated blood, followed by anticoagulation with citrate and EDTA, indicating a role for calcium in the process of cytokine production and release. A similar result was obtained for intracellular cytokine evaluation by flow cyotmetry, i.e. the frequency of labeled cells was increased in heparin treated samples. The method of anticoagulation, however, did not affect the results of RT-PCR over a 1 hr period, as levels of mRNA were not significantly different between methods of anticoagulation following heparin removal and the present results agree with a previous study[10]

Superficially, it appeared that intracellular flow cytometry was the most sensitive method for assessing cytokine synthesis by leukocytes. However, it took two hours to accumulate sufficient cytokine to be detectable by flow cytometry. RT-PCR has been shown capable of detecting mRNA in single cells. In the present study we did not push the sensitivity to that limit because we were interested in the average synthesis by a heterogenous population of leukocytes and were estimating mRNA from about 200 cells. Since we could detect increased mRNA synthesis within 30 minutes, in the present study, the two techniques were roughly comparable in sensitivity.

Based on ELISA and cytokine flow cytometry for TNF-α, IL-lα, IL-lβ, and IL-8, synthesis and release was correlated to mRNA expression. The principal leukocytes producing these cytokines were monocytes and granulocytes. That the granulocyte is an important

contributor to TNF-α production was also noted by Dubravec et al,[11] after careful isolation of granulocytes and monocytes. Their results also indicated that monocytes produced about three times as much TNF-α as granulocytes, although this system used a large dose of LPS (5 ug/ml) and required long incubation times to see relatively small increases. Here, we additionally show that IL-lα and IL-lβ, and IL-8 are produced by both monocytes and granulocytes.

Concentrations as low as 10 pg/ml of LPS stimulated a measurable cytokine response. This is a concentration four-to-five orders of magnitude lower than has been used in most studies. At 1 hr post LPS treatment, comparable concentrations of LPS resulted in both TNF-α message elevation and cytokine release. TNF-α mRNA was elevated slightly above the baseline after whole blood incubation with 10 pg/ml for I hr, and its product was also released in low amounts. With 100 pg/ml LPS stimulation, TNF-α was released in quantities of over 2,000 pg/ml of plasma, comparable to the quantity found after whole blood incubation with 1 mg/ml LPS.

For IL-8 released into the plasma, the amount of LPS required for cytokine release was ten-fold higher than that required for mRNA transcription after 1 hr incubation with LPS. With 100 pg/ml of LPS, there was a release of 10 pg/ml of IL-8. This was more than two orders of magnitude lower than IL-8 release following incubation with 1 ng/ml concentrations of LPS. Ten pg/ml of LPS yielded little measurable IL-8 product in the medium, but message for this cytokine was elevated above the baseline.

IL-lα and IL-1β synthesis was correlated with TNF-α synthesis, but its levels did not increase in the plasma. The in vivo response of IL-1 release is well characterized. It is a component of the acute phase response, and co-stimulatory with TNF-α. In the present study, there were never measurable amounts of IL-lα and IL-lβ in the plasma after LPS treatments of 30 min or 1 hr. Thus, IL-lα and IL-lβ release appear to be mediated by some factor(s) not accounted

for in isolated whole blood exposed to endotoxin. The activation of the interleukin 1 converting enzyme is known to be required to process the procytokine before secretion.[12] This additional processing requirement may explain the failure to detect significant amounts of IL-1β in the medium.

LPS stimulation of cytokine synthesis and release from whole blood incubated in vitro provides a model that exhibits many aspects of septic shock in vivo. It also allows ready analysis of gene expression at both the level of transcription and translation and should continue to provide an important in vitro model for the cellular response to LPS.

Estimations of the frequency of labeled cells and mean channel fluorescence by flow cytometry provides an additional important new means for examining cytokine expression. The high inherent throughput capability of flow cytometry allows the study of individual cells directly ex vivo, minimizing artifacts due to culture. As such, flow cytometry studies provide unique insights into cytokine biology not possible previously.[6] Flow cytometry allowed us to confirm that granulocytes also synthesized TNF-α, IL-1α and -1β, and IL-8.

LEGENDS

Figure 1. Dependence of release of cytokines (TNF-α, IL-8 and IL-1β) from whole blood on stimulating dose of LPS as determined by ELISA. Whole blood was incubated with 0 (open bar), 10 pg (gray bar), 100 pg (hatched bars), or (closed bar) 1000 pg of LPS. Blood was collected in citrate, EDTA or heparin and incubated for 2 hr at 37°C. Plasma was obtained by centrifugation of whole blood at 8,000 x g for 5 minutes and cytokines measured by ELISA. Data presented as mean ± SEM.

Figure 2. RT-PCR inhibition in whole blood collected into heparin. RT-PCR products from whole blood visualized in a SYBR Green I stained agarose gel of lymphocytes collected into citrate, EDTA or heparin without and with isolation of cells before reverse transcription and PCR. G3PDH mRNA target amplifies at 983 base pairs. The 630 base pair band is the amplified mimic DNA. The reverse transcriptase product is inhibited as indicated by absence of band for target (arrows) and interference in PCR is indicated by diminished mimic bands (hands).

Figure 3. Effect of LPS dose on TNF-α (A) IL-lα (B) IL-8 (C),and IL-lβ (D) mRNA transcription. PCR was performed with 10^{-2} attomoles of mimic for TNF-α and IL-8 and 10^{-3} attomoles of IL-lα mimic per 10 μL reaction. IL-lβ was performed without mimic. LPS was incubated with heparinized whole blood for 1 hour. RNA was extracted from triplet cell pellets and RT-PCR performed on each. The data are presented as mean ± SEM. *$p \leq 0.05$ versus control (0 pg LPS).

Figure 4. Influence of anticoagulation and separation of leukocytes prior to RT-PCR on TNF-α production. Blood was collected into citrate, EDTA, or heparin and stimulated with 1 μg/ml LPS for 1 hr at 37°C. The TNF-α target is 444 bp while the mimic is 617 bp.

Figure 5. (A) TNF-α and (B) IL-8 mRNA transcription levels after LPS incubation for 30 minutes and 1 hr. Transcription levels determined by RT-PCR after 1 hr at 37°C in heparinized blood. PCR was performed with 10^{-2} attomoles of IL-8 and TNF-α mimic per 10 μl reaction. RNA was extracted from a total cell pellet, obtained by centrifuging whole blood at 8,000 g for 5 minutes, after which time the plasma was drawn off and cells were lysed in TRI-LS.

Figure 6. Cytokine flow cytometry analysis of human whole blood. Samples were analyzed by 3-color flow cytometry. Gating was based on side scatter vs cell surface marker expression. A single gate was set on a selected lymphocytic population defined by a region (R) specific to a cell surface marker. (A) Side scatter vs. CD3+(T-cells)R1, (B) Side scatter vs. CD14+(Monocytes)R2, (C) Side scatter vs CD16+ (Granulocytes)R3, (D) Side scatter vs CD19+(B-cells)R4. Isotypic controls for both the intracellular cytokine and the cell surface markers were used to determine the positioning of the threshold for negative and positive events. Data are expressed as percent positive cells for cytokine (IL-1β, TNF-α and IL-8) positive cells as a percentage of each gated sub-population.

Figure 7. Cytokine flow cytometry analysis of heparinized human whole blood. Whole blood was incubated with 0 (not shown), 100 pg (open bar), or 1000 pg (hatched bar) of LPS for 2

hours. Samples were gated and analyzed by 3-color flow cytometry as described in Figure 6.

Figure 8. Cytokine flow cytometry analysis of heparinzed human whole blood. Data shown are expressed as mean fluorescence channel value of each cytokine (IL-lβ, TNF-α, IL-8) above the control background fluorescence for each specific cell surface marker and gated population. Whole blood was incubated with 0 (open bar), 100 pg (hatched bar), or 1000 pg (closed bar) of LPS for 2 hrs. Samples were gated and analyzed by 3-color flow cytometry as described in Figure 6.

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Anticoagulant	Extracellular Calcium	Normalization to Heparin
Citrate	1.94 +/- 0.44 mM	0.86
EDTA	0.62 +/- 0.49 mM	0.27
Heparin	2.25 +/- 0.1 mM	1

Table I. The affect of anticoagulant on extracellular calcium concentration and change relative to heparin.

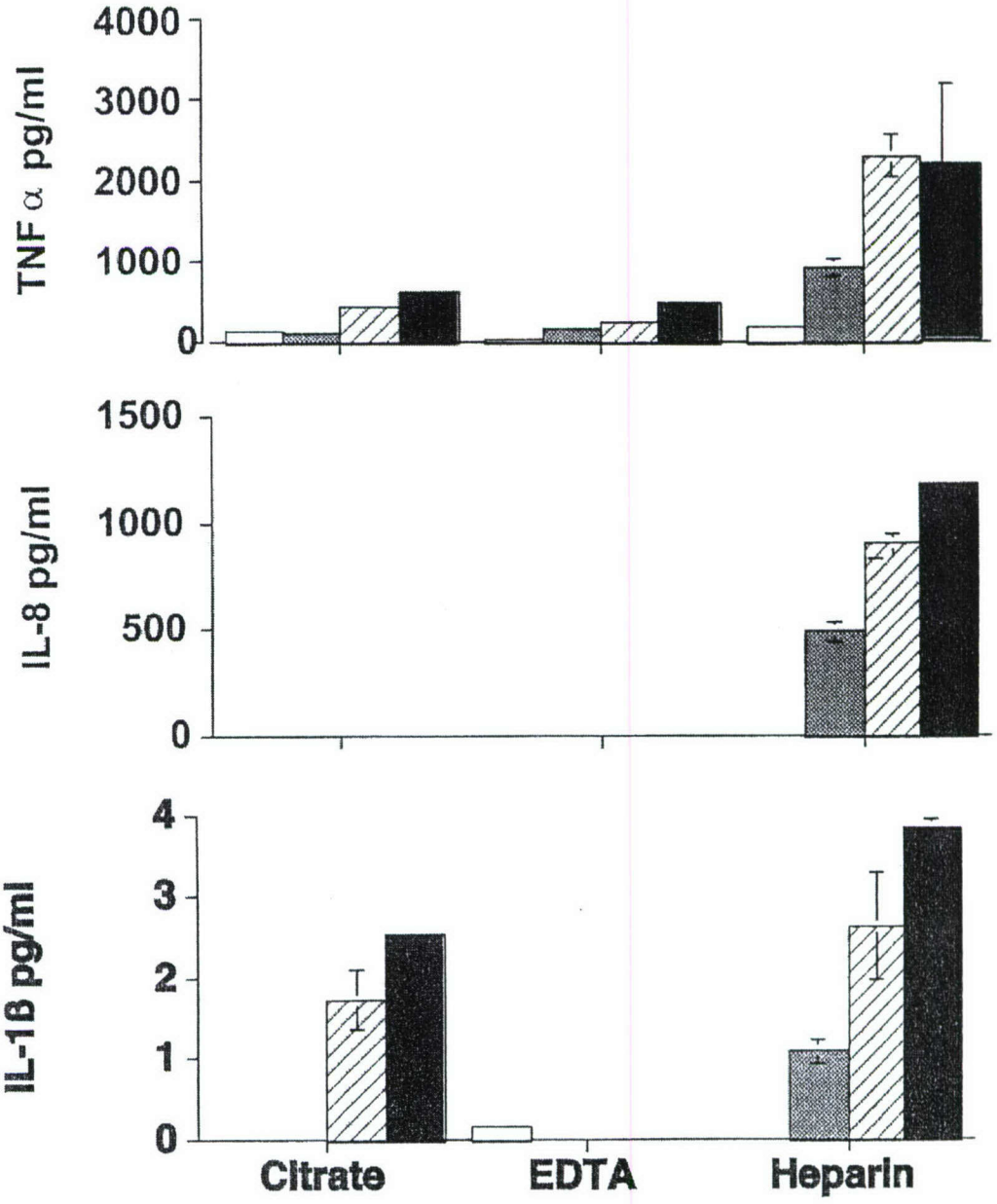
TNF α pg/ml
4000
3000
2000
1000
0
IL-8 pg/ml
1500
1000
500
0
IL-1B pg/ml
4
3
2
1
0
Citrate
EDTA
Heparin

Figure 1

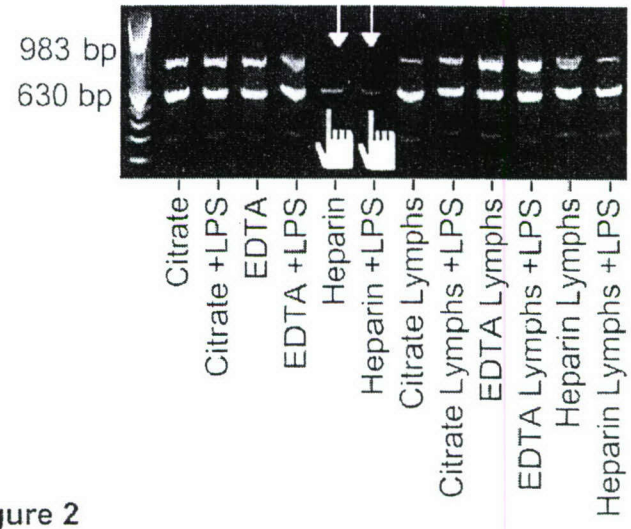
983 bp
630 bp
Citrate
Citrate +LPS
EDTA
EDTA +LPS
Heparin
Heparin +LPS
Citrate Lymphs
Citrate Lymphs +LPS
EDTA Lymphs
EDTA Lymphs +LPS
Heparin Lymphs
Heparin Lymphs +LPS

Figure 2

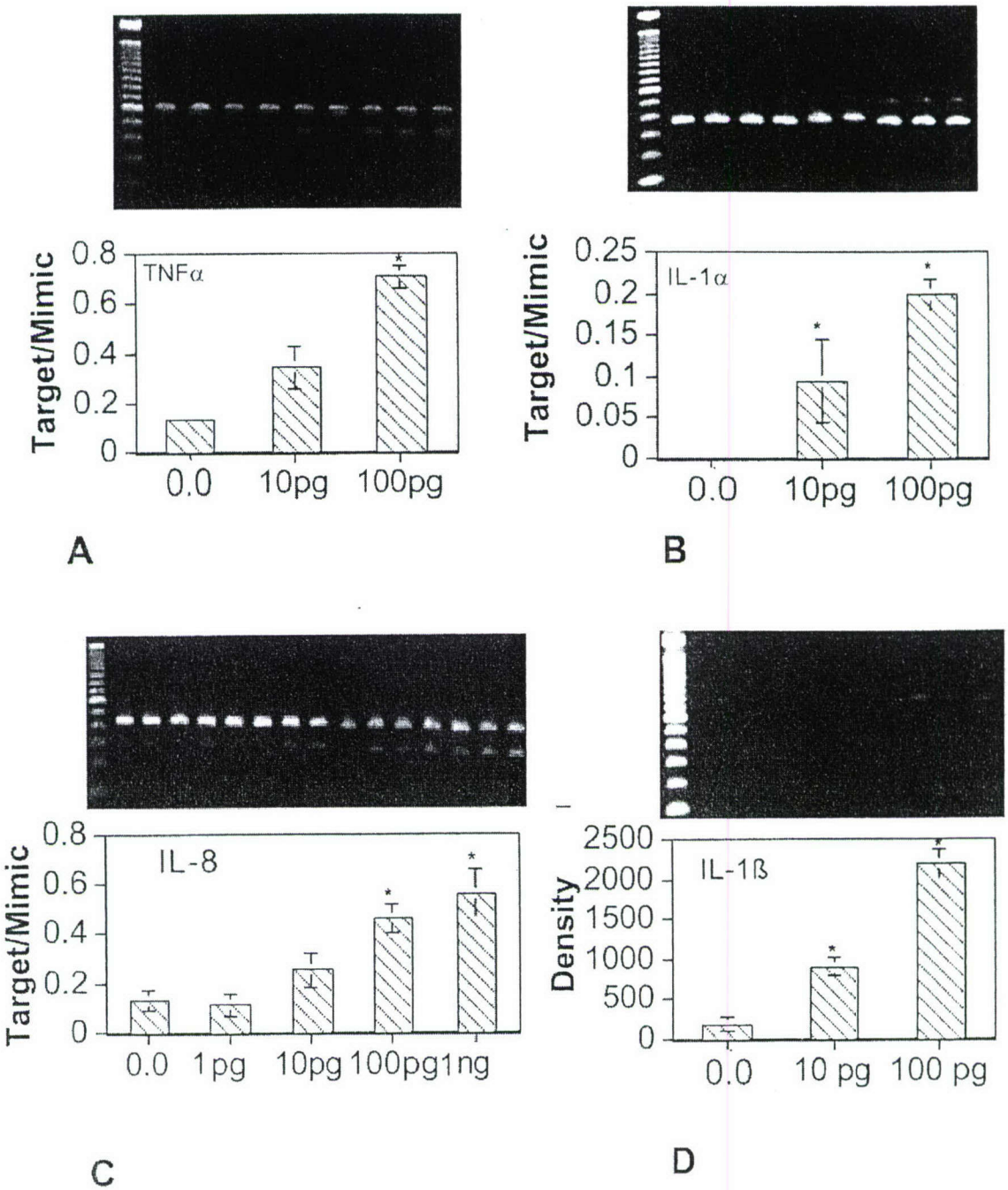
TNFα
Target/Mimic
0.8
0.6
0.4
0.2
0
0.0
10pg
100pg
A
IL-1α
Target/Mimic
0.25
0.2
0.15
0.1
0.05
0
0.0
10pg
100pg
B
IL-8
Target/Mimic
0.8
0.6
0.4
0.2
0
0.0
1pg
10pg
100pg
1ng
C
IL-1ß
Density
2500
2000
1500
1000
500
0
0.0
10 pg
100 pg
D

Figure 3

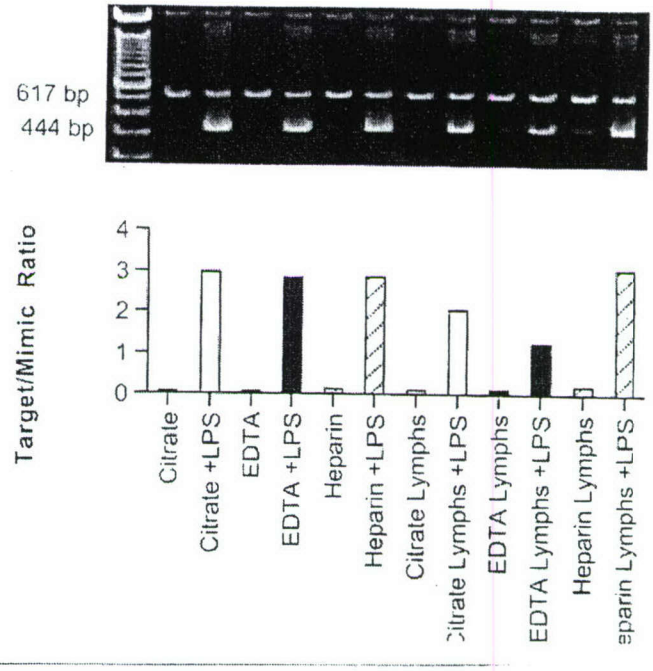
617 bp
444 bp
Target/Mimic Ratio
4
3
2
1
0
Citrate
Citrate +LPS
EDTA
EDTA +LPS
Heparin
Heparin +LPS
Citrate Lymphs
Citrate Lymphs +LPS
EDTA Lymphs
EDTA Lymphs +LPS
Heparin Lymphs
eparin Lymphs +LPS

Figure 4

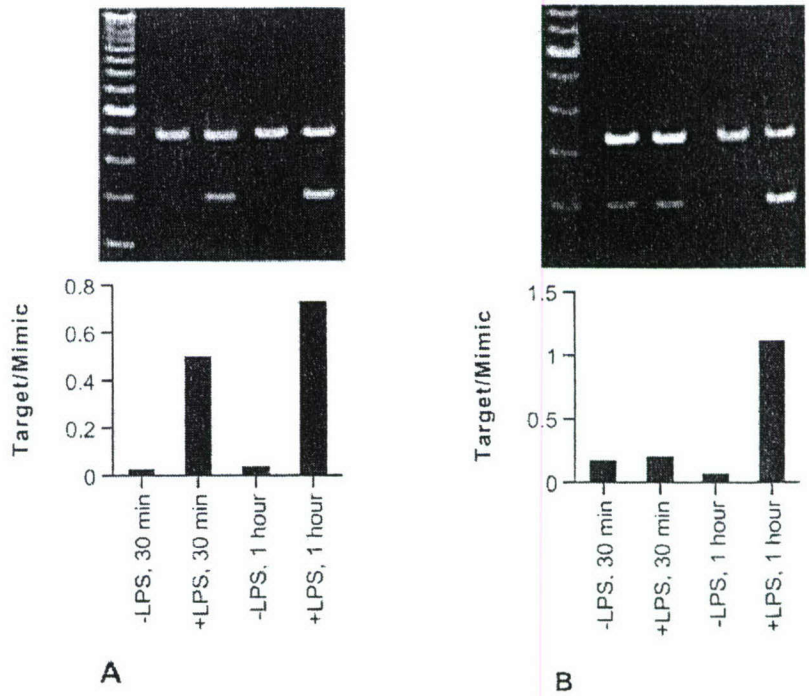
Target/Mimic
0.8
0.6
0.4
0.2
0
-LPS, 30 min
+LPS, 30 min
-LPS, 1 hour
+LPS, 1 hour
A
Target/Mimic
1.5
1
0.5
0
-LPS, 30 min
+LPS, 30 min
-LPS, 1 hour
+LPS, 1 hour
B

Figure 5

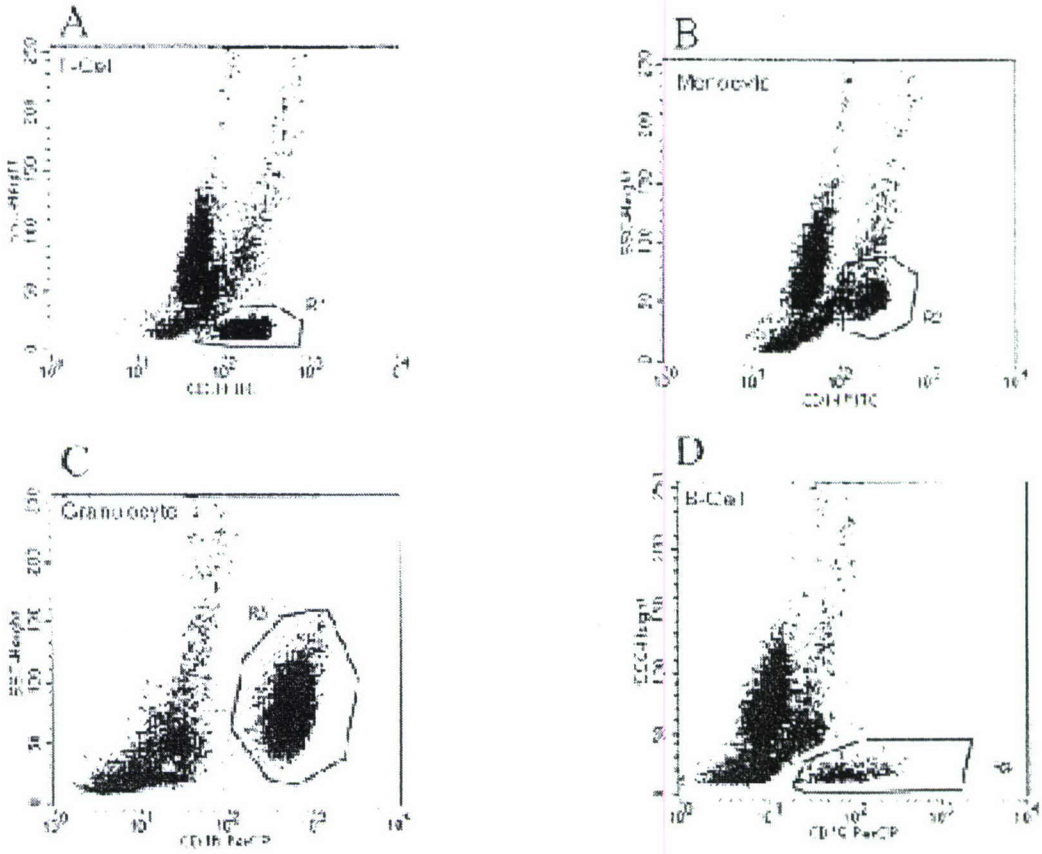

Figure 6

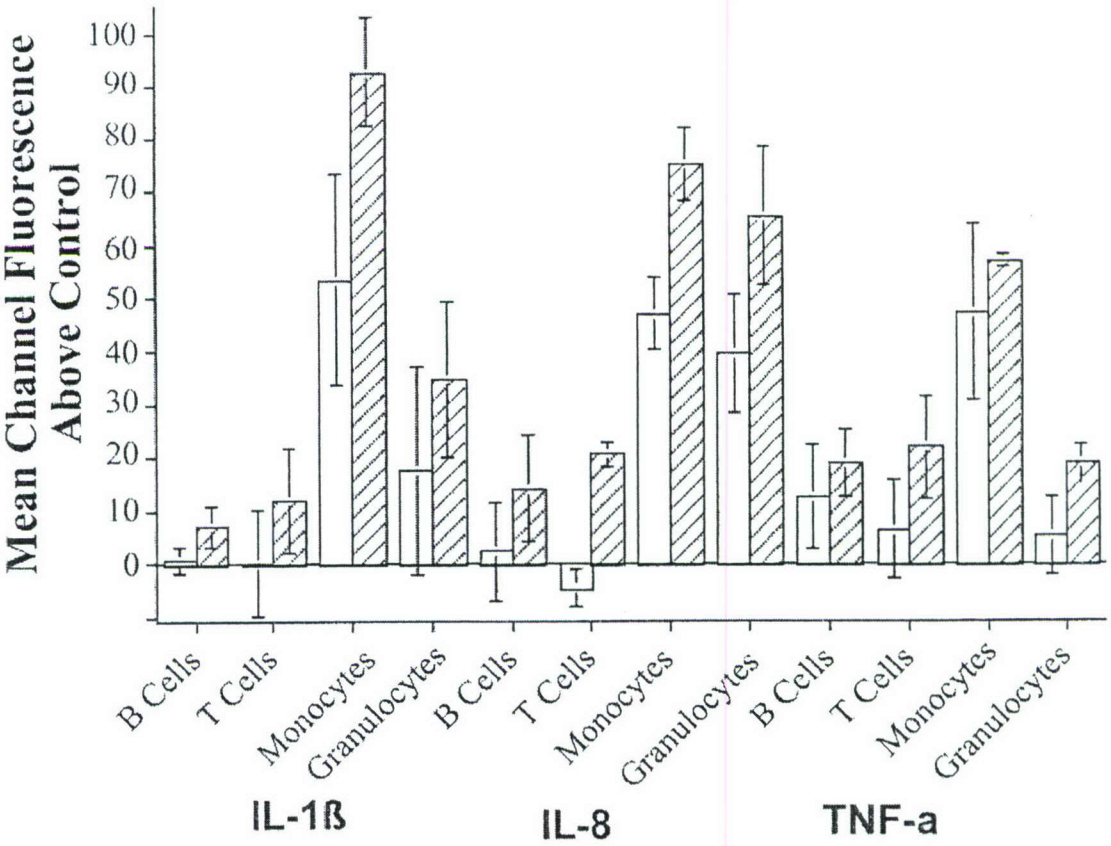
Mean Channel Fluorescence Above Control
100
90
80
70
60
50
40
30
20
10
0
B Cells
T Cells
Monocytes
Granulocytes
B Cells
T Cells
Monocytes
Granulocytes
B Cells
T Cells
Monocytes
Granulocytes
IL-1ß
IL-8
TNF-a

Figure 7

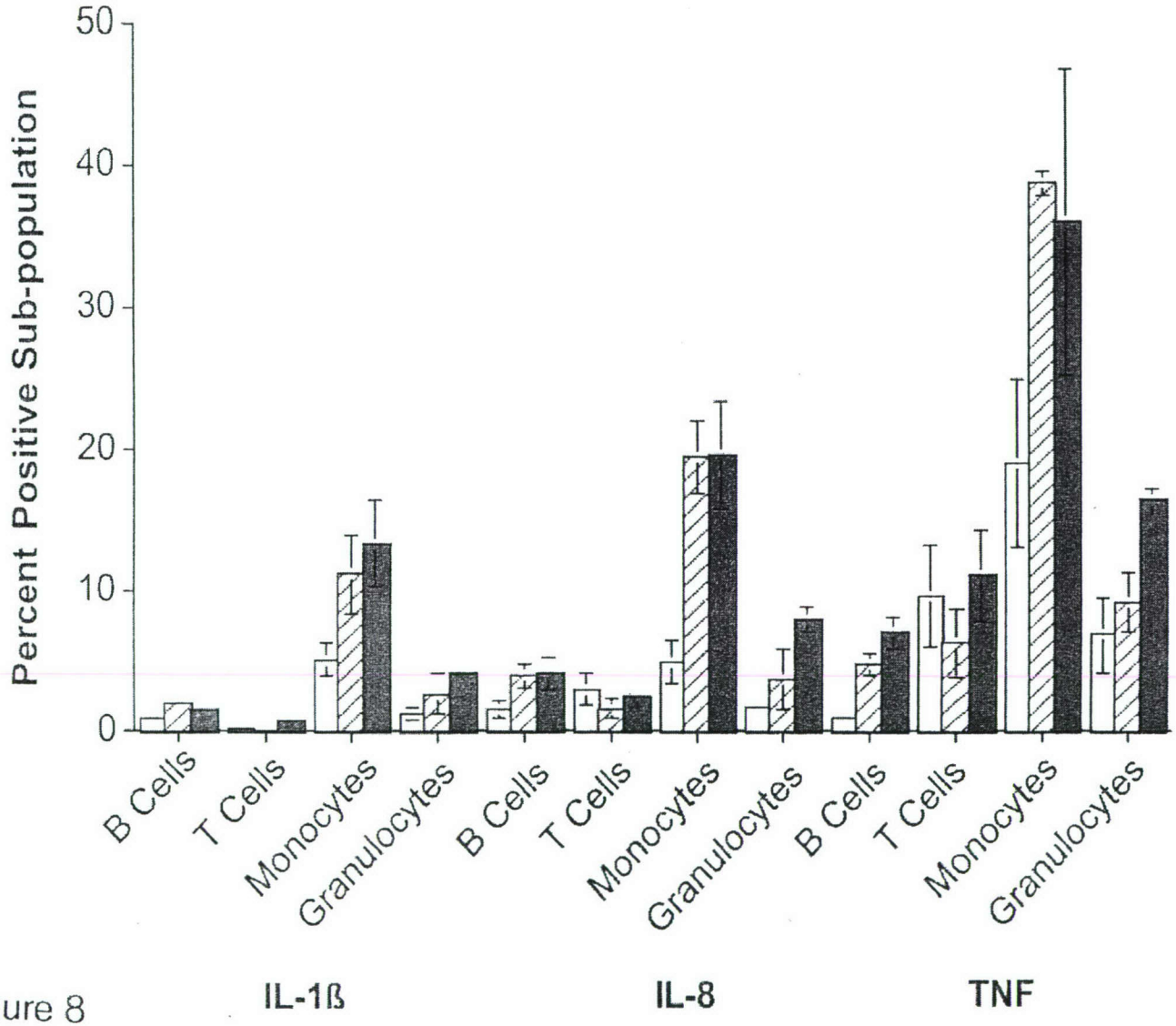
Percent Positive Sub-population
50
40
30
20
10
0
B Cells
T Cells
Monocytes
Granulocytes
B Cells
T Cells
Monocytes
Granulocytes
B Cells
T Cells
Monocytes
Granulocytes
IL-1ß
IL-8
TNF

Figure 8